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# c-Rel-Dependent Priming of Naive T Cells by Inflammatory Cytokines

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## Summary

**The intrinsic refractoriness of naive T cells for cytokine production is counteracted by cells of the innate immune system. Upon sensing danger via Toll-like receptors, these cells upregulate T cell costimulatory molecules and secrete cytokines that enhance T cell activation. We show that cytokine-mediated priming of naive T cells requires the NF-κB family member c-Rel. In resting naive cells c-Rel is associated primarily with IκBβ, an inhibitory molecule that is not effectively degraded by TCR signals. Exposure of T cells to proinflammatory cytokines, TNF-α and IL-1β, shifts c-Rel to IκBα-associated complexes that are readily targeted by the TCR. As a consequence, IL-2 and IFN-γ mRNA are produced more quickly, and at higher levels, in cytokine-primed T cells. This mechanism does not operate in effector T cells where cytokine gene expression is c-Rel-independent. We propose that c-Rel plays a crucial role as a target of innate signals in T cells.**

## Introduction

The NF-κB family of transcription factors consists of homodimers and heterodimers of Rel-homology domain (RHD)-containing proteins (Bonizzi and Karin, 2004; Hayden and Ghosh, 2004). Two small subunits, p50 and p52, are generated from larger precursor proteins by proteolytic processing. These subunits contain few additional residues beyond the approximately 300 amino acid RHD, and their homodimers are constitutively nuclear. The three larger subunits, p65/RelA, c-Rel, and RelB, contain transcription regulatory domains in addition to the RHD. Subcellular distribution, as well as DNA binding, of complexes that contain these Rel subunits is regulated by interaction with inhibitory IκB proteins. In most unactivated cells, Rel/IκB complexes are detected primarily in the cytoplasm, suggesting a model in which IκB proteins sequester Rel proteins in the cytoplasm. However, accumulating evidence indicates that Rel proteins complexed to IκBα shuttle continuously between the nucleus and cytoplasm of unstimulated cells. Thus, the predominantly cytosolic distribution of these complexes simply reflects a "snapshot" of a dynamic state. Nuclear import of Rel/IκB complexes is probably mediated by nuclear-localization sequences in the Rel proteins, whereas retrieval from the nucleus is mediated by nuclear-export sequences in IκBα and p65/RelA (Huang et al., 2000; Tam et al., 2000; Tam et al., 2001) . Other IκBs do not contain efficient nuclear-export sequences (Lee and Hannink, 2002; Malek et al., 2001; Tam and Sen, 2001) and probably regulate Rel subcellular distribution by the sequestration mechanism.

Studies of lymphocytes that lack one or more Rel subunits show that these proteins play important and nonredundant roles in immune function (Li and Verma, 2002). Even the closely related p65/RelA and c-Rel proteins serve different functions in B and T lymphocytes. For example, c-Rel-deficient B cells die rapidly in response to antigen receptor crosslinking without entering the cell cycle (Grumont et al., 1999; Grumont et al., 1998; Hsia et al., 2002; Owyang et al., 2001; Yamazaki and Kurosaki, 2003). This is attributed to diminished transcriptional induction of Bcl-2 family anti-apoptotic genes Bfl.A1 and BclxL. In contrast, c-Rel-deficient T cells do not die upon TCR crosslinking, although there is a block in proliferation that can be rescued by exogenous interleukin-2 (IL-2) (Kontgen et al., 1995; Liou et al., 1999). The major defect in c-Rel-deficient T cells is diminished cytokine production, although the extent of the defect varies considerably in the published literature, in part depending on the stimulus used (Hilliard et al., 2002; Kontgen et al., 1995; Liou et al., 1999; Rao et al., 2003). A lack of p65/RelA induction in response to TCR stimulation leads to rapid cell death, reminiscent of the phenotype of c-Rel-deficient B cells (Wan and DeGregori, 2003). However, different mechanisms may operate in the two cell types because T cell death in the absence of NF-κB has been attributed to activation of the p53 homolog, p73.

Unactivated B and T lymphocytes differ in the composition of NF-κB/IκB complexes in one major way. Whereas p65/RelA protein is complexed to IκBα and IκBβ in both cell types, c-Rel is complexed primarily to IκBβ in T cells (Tam et al., 2001). Thus, IκBα-degrading signals, such as those initiated at the T cell receptor or its pharmacologic equivalent, induce only p65/RelA by the classical post-transcriptional pathway of NF-κB activation (Venkataraman et al., 1996). Nuclear c-Rel expression requires new gene transcription and translation by a pathway that is suppressed by FK506 or cyclosporin A (Venkataraman et al., 1995, 1996; Grumont et al., 2004). This mechanism results in slower c-Rel induction and, as a consequence, slower c-Rel-dependent gene transcription compared to p65/RelA-dependent genes that are rapidly activated. Signals initiated at the B cell antigen receptor target both IκBα and IκBβ, which leads to rapid post-translational induction of p65/Rel A as well as c-Rel (Venkataraman et al., 1996).

Because such features of NF-κB regulation may explain phenotypic aspects of the responses of activated

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lymphocytes, we further examined the implications of c-Rel/IκB association in T cells. We found that the proportion of c-Rel associated with IκBα increased substantially in cells pre-activated with TNF-α or interleukin-1 (IL-1). This was due to the shuttling property of newly synthesized IκBα, which migrated into the nucleus and exported nuclear c-Rel out to the cytoplasm. Consequently, T cells that had previously seen cytokines responded to IκBα-degrading signals initiated at the TCR by rapidly activating c-Rel nuclear translocation, and c-Rel-dependent cytokine gene expression was significantly increased. These observations suggested that inflammatory-cytokine-induced alteration of c-Rel/IκB complexes may prime naive T cells to TCR signals. Consistent with this hypothesis, we found that T cells from CFA-primed wild-type, but not c-Rel-deficient, mice responded robustly to TCR stimulation by inducing high levels of interleukin-2 (IL-2) and interferon-γ (IFN-γ) gene transcription. Interestingly, c-Rel-mediated priming did not occur in effector T cells, in which IL-2 and IFN-γ gene expression was c-Rel independent. We propose that the shift of c-Rel to IκBα in T cells induced by inflammatory cytokines constitutes one form of communication between cells of the innate and adaptive immune systems during the generation of a primary immune response.

## Results

### c-Rel/IκBα Complex Formation in Response to Inflammatory Cytokines

Unactivated D5h3 T hybridoma cells contain predominantly c-Rel/IκBβ complexes, whereas p65/RelA is associated with both IκBα and IκBβ. Acute treatment with TNF-α induced IκB degradation (Figure 1A, left panel) and nuclear translocation of c-Rel and p65/RelA (Figure 1A, right panel). TNF-α withdrawal was accompanied by resynthesis of both IκBs and loss of nuclear NF-κB to regenerate the cytoplasmic pool of IκB-associated Rel proteins (Figure 1A). However, cells that were pretreated with TNF-α differed from unactivated cells in that they contained significant levels of c-Rel/IκBα complexes as assayed by immunoprecipitation followed by immunoblotting (Figure 1A, lower panel, lanes 6–9). The proportion of p65 or c-Rel bound to IκBβ versus IκBα is indicated below the gel and shows that 6 hr after TNF-α withdrawal approximately 30% of cytosolic c-Rel was associated with IκBα compared to less than 5% in untreated cells. Because the total amount of c-Rel did not change over the experimental time course (Figure 1A), the absolute amount of c-Rel/IκBα complexes increased after cytokine treatment. c-Rel shift to IκBα was most likely due to newly synthesized IκBα, which migrated into the nucleus, bound Rel proteins, and exported them to the cytoplasm. Consistent with this idea, inclusion of leptomycin B to block nuclear export prevented c-Rel localization to the cytoplasm (data not shown). The slower resynthesis of IκBβ (Figure 1A, top left panel) accentuated the role of IκBα during this phase of NF-κB downregulation from the nucleus.

To determine if continued TNF-α signaling also resulted in a shift of c-Rel to IκBα, we treated D5h3 T hybridoma cells with different amounts of TNF-α for 6 hr and then analyzed IκB/Rel complexes by coimmunoprecipitation. Although c-Rel was primarily associated with IκBβ in unactivated cells (Figure 1B, lanes 2-4), significant c-Rel/ IκBα complexes were detected in cells treated with low levels of TNF-α (Figure 1B; compare lanes 5, 7 and 9 to lane 3). A similar shift to IκBα association was evident in primary CD4+ T cells in response to TNF-α (Figure 1C; compare the proportion c-Rel on IκBα and IκBβ in lanes 3 and 4 to that in lanes 5 and 6). Another NF-κB-inducing cytokine, interleukin 1-β (IL-1β), as well the combination of TNF-α plus IL-1, also increased the number of c-Rel/IκBα complexes even more effectively than TNF-α alone (Figure 1C; compare lanes 7–10 to lanes 3 and 4). We verified the efficiency and specificity of IκBβ immunoprecipitation by assaying the immunoprecipitation supernatants for the appropriate proteins (Figure S1B in the Supplemental Data available with this article online). Because this shift occurred without significant alteration in the levels of either Rel or IκB proteins (Figure S1A), we conclude that proinflammatory cytokines such as TNF-α and IL-1β increase the overall levels of c-Rel/IκBα complexes in T cells. Consistent with the increase in c-Rel/IκBα complexes in cytokine-treated cells, we observed more efficient c-Rel induction in response to anti-CD3 crosslinking, which is primarily an IκBα degrading signal (Figure S1C).

### Cytokine Gene Expression in TNFα/IL-1β Pretreated Cells

Inflammatory cytokines, such as TNF-α, IL-1, and IL-6 have been previously shown to enhance T cell proliferation in vivo and in vitro (Curtsinger et al., 1999; Khoruts et al., 2004; Kurt-Jones et al., 1987; Pape et al., 1997; Rojo et al., 1989; Teague et al., 2000). These cytokines have also been recently shown to overcome age-related deficiency of naive CD4+ T in the production of interleukin-2 (IL-2) (Haynes et al., 2003; Haynes et al., 2004). Because c-Rel has been previously implicated in IL-2 gene expression, our observation that TNF-α and IL-1 shifted c-Rel to rapidly activatable IκBα-associated complexes provided a possible mechanism for the costimulatory activity of these cytokines. To test this, we examined the effect of TNF-α and IL-1 on cytokine gene expression in splenic CD4+ T cells from normal and c-Rel-deficient mice.

Purified CD4+ T cells were treated with the cytokines for 6 hr and subsequently stimulated with low levels of plate-bound anti-CD3 antibody. IL-2 and IFN-γ expression was scored by quantitative RT-PCR with total RNA isolated after various times of CD3 activation. We observed 3- to 5-fold higher IL-2 and IFN-γ mRNA expression in normal cells that had been treated with IL-1 alone or with IL-1 plus TNF-α (Figure 2A). TNF-α alone did not affect gene expression significantly in these assays despite its being able to generate c-Rel/IκBα complexes. We surmise this may be because of more pleiotropic effects of TNF-α on T cells (Clark et al., 2004; Isomaki et al., 2001), including effects on TCR signaling (Church et al., 2005). At this level of stimulation, IL-2 and IFN-γ induction in c-Rel$^{-/-}$ CD4+ T cells was comparable to that seen in unprimed normal CD4+ T cells

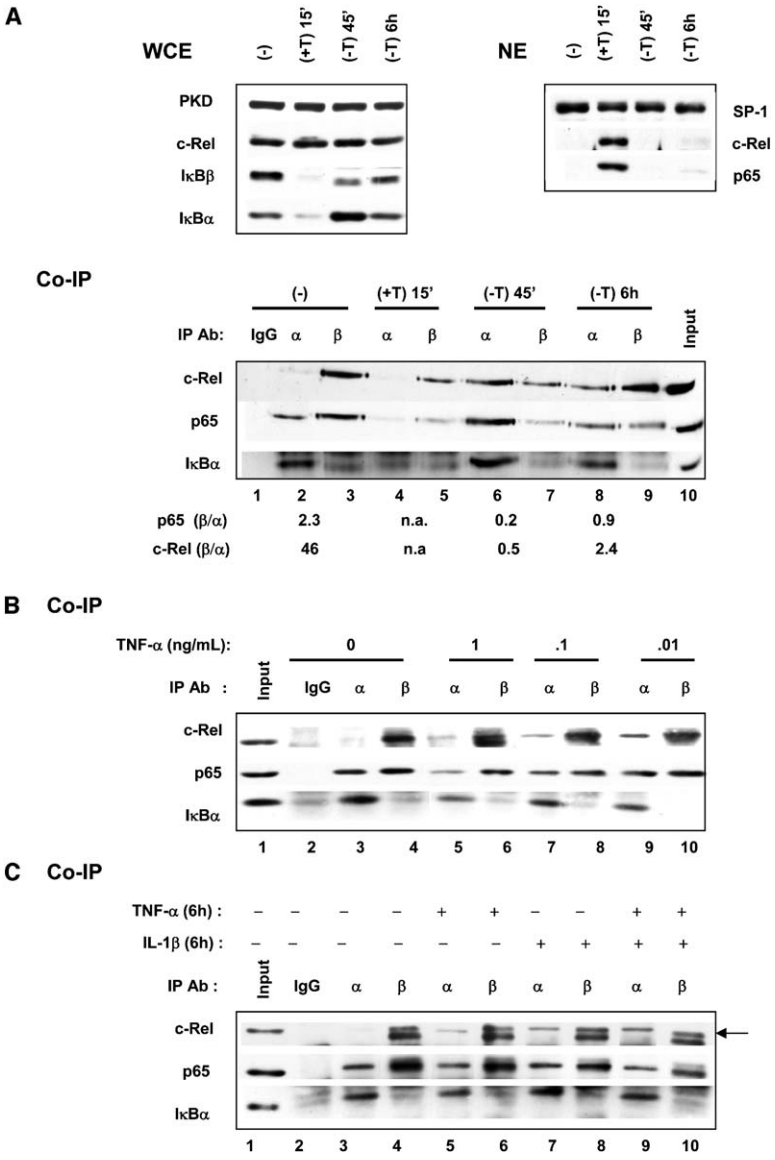


Figure 1. Pro-Inflammatory Cytokines Induce Formation of c-Rel/IκBα Complexes in T Lymphocytes

(A) c-Rel/IκBα complex formation with acute TNF-α stimulation. D5h3 T hybridoma cells were treated with TNF-α (10 ng/ml) for 15 min ([+T] 15′) and then incubated for various times in the absence of the cytokine (–T). Whole-cell extracts (WCE) and nuclear extracts (NE) prepared from these cells were fractionated by SDS-PAGE and immunoblotted with antibodies against p65, c-Rel, and IκBα and IκBβ molecules as indicated. Protein kinase D (PKD) and the transcription factor SP1 were used to confirm comparable protein loadings between extracts. Results shown are representative of more than six independent experiments. WCE were used for coimmunoprecipiation studies (Co-IP) (bottom panel). The origin of extracts is noted on the top line above the figure, and the immunoprecipitating antibody (IP Ab) is noted on the second line; IgG represents non-specific rabbit IgG, and α and β represent anti-IκBα and anti-IκBβ antibodies, respectively. The immunoprecipitates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes, which were probed with anti-p65/RelA, anti-c-Rel, and anti-IκBα antibodies as indicated to the left of the panel. The proportion of p65/RelA, or c-Rel, bound to IκBβ compared to IκBα was quantified as described in the Experimental Procedures and is indicated below the gels. The data is representative of three independent experiments.

(B) c-Rel/IκBα complex formation with continuous TNF-α stimulation. T hybridoma cells were treated with various levels of TNF-α (shown on the top line) for 6 hr followed by preparation of whole-cell extracts. c-Rel or p65/RelA associated with IκBα or IκBβ was determined by coimmunoprecipiation as described in the legend to (A). Data shown are representative of three independent experiments.

(C) c-Rel/IκBα complex formation in primary CD4+ T cells. CD4+ T cells, purified by positive selection with anti-CD4-coated magnetic beads, were treated with TNF-α (5 ng/ml), interleukin 1β (IL-1β, 5 ng/ml), or both together for 6 hr, followed by preparation of whole-cell extracts. c-Rel or p65/RelA association with IκBα was determined by coimmnoprecipitation experiments as described in the legend to (A). The top arrow indicates the position of c-Rel; the lower band in anti-IκBβ immunoprecipitates has not been characterized. Data shown are representative of three independent experiments.

(additional bars to the right of the time course in Figure 2B), indicating that c-Rel is not essential for inducible transcription of these genes. However, TNF-α- and IL-1-treated c-Rel$^{-/-}$ CD4+T cells did not express higher levels of IL-2 or IFN-γ mRNA, indicating that c-Rel was essential for the "super-induction" seen in normal cells pretreated with TNF-α and IL-1.

These observations are consistent with a mechanism whereby increased levels of c-Rel/IκBα complexes in TNF-α- and IL-1-treated cells enhanced the subsequent responsiveness of these cells to stimulation via the TCR. We hypothesized that these complexes are generated by export of nuclear c-Rel by newly synthesized IκBα. To further corroborate a role for nuclear export and cytoplasmic reactivation, we used IκBα mutants that either lacked the N terminus (and thus could not be targeted by IκB kinases) or inactivated an N-terminal nuclear-export sequence (NES) that severely reduced its export-chaperone ability (Huang et al., 2000; Huang and Miyamoto, 2001; Tam et al., 2001). For reasons outlined below (see Figure 6 and accompanying discussion), these experiments were carried out in D5h3T hybridoma cells. We expressed IκBα mutants by using retroviral gene transfer and compared cytokine gene expression in sorted GFP+ (infected) and GFP− (uninfected) cells that had been pretreated with TNF-α, or not (the fact that D5h3 hybridoma cells were refractory to IL-1 precluded its use in these studies).

TNF-α pretreatment resulted in approximately 2.5-fold-enhanced IL-2 mRNA expression in response to anti-CD3 in uninfected cells (Figure 3A, top panel). This priming effect was abolished in cells that expressed

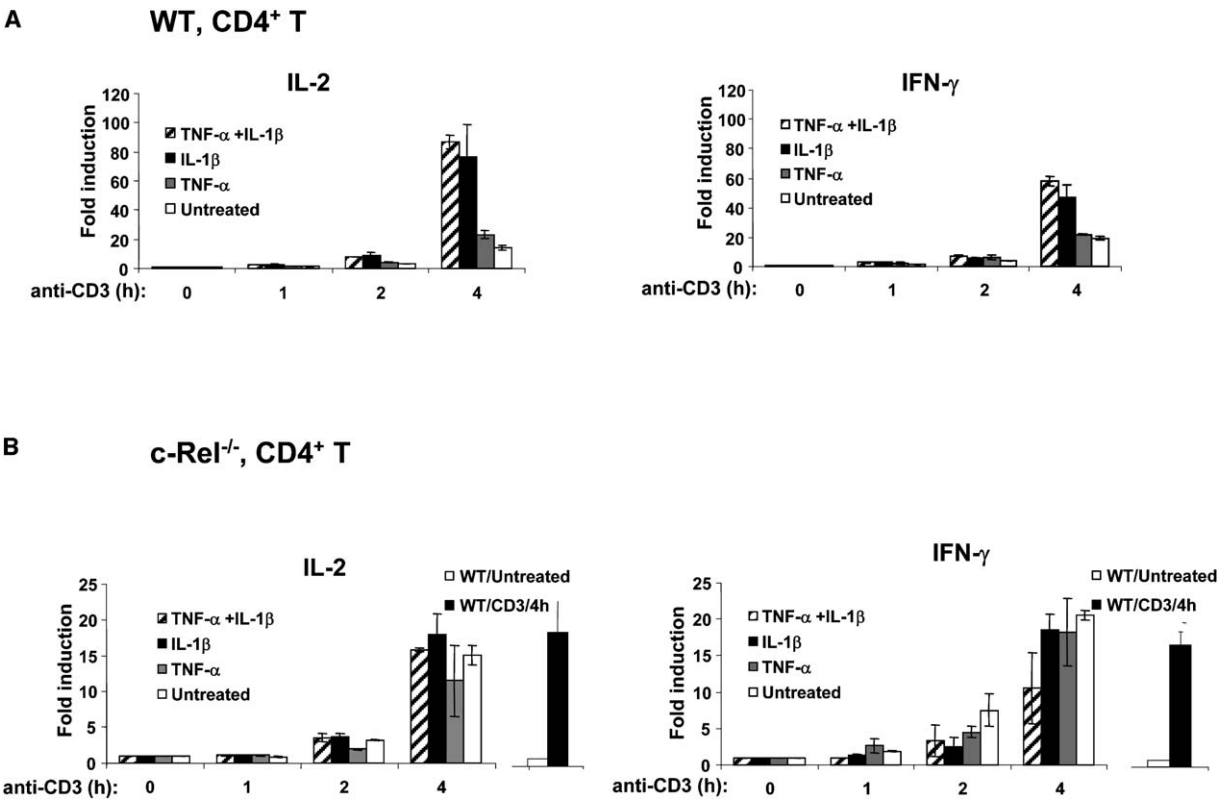


Figure 2. TNF-α and IL-1β Prime Normal, but Not c-Rel-Deficient, CD4+ T Cells for Increased Cytokine mRNA Production

Purified splenic CD4+ T cells from normal (A) or c-Rel-deficient (B) mice were treated continuously for 6 hr with TNF-α, IL-1β, or TNF-α and IL-1β in-vitro, and activation with plate bound anti-CD3 antibodies (0.5 μg/ml) followed for the times indicated (*x* axis). Total RNA prepared from the cells was examined for IL-2 (left panel) and IFN-γ (right panel) expression via quantitative RT-PCR as described in the Experimental Procedures section. The signals were normalized to TCRβ mRNA and represented as "Fold Induction" (*y* axis) over the basal level detected in cells prior to CD3 activation. RNA assays were carried out in duplicate, and error bars shown represent the standard error within the duplicate. For ease of comparison, additional bars marked WT/untreated in (B) show the level of cytokine mRNA in normal cells not treated with TNF-α or IL-1β as an average of three to four independent experiments. Data shown are representative of two independent experiments with normal and c-Rel-deficient cells.

both forms of mutated IκBα genes. ΔN-IκBα-expressing cells produced somewhat lower levels of IL-2 after TNF-α pretreatment (Figure 3A, middle panel), whereas the effect was greatly diminished in NES$^{-}$-IκBα-expressing cells (Figure 3A, bottom panel). The residual enhancement seen with NES$^{-}$-IκBα may be due to a weaker NES at the IκBα C terminus (Ossareh-Nazari et al., 1997), which remains intact in our mutant. We conclude that IκBα-dependent nuclear export and reactivation in the cytoplasm are essential to prime T cells for enhanced IL-2 gene expression.

To further assess the role of IκBα in priming splenic CD4+ T cells, we used siRNA to downregulate endogenous IκBα. IκBα or a control siRNA was introduced into primary CD4+ T cells by electroporation, resulting in 2-fold reduction of steady-state IκBα mRNA measured by RT-PCR (Figure 3B, left panel). To determine the effect on IκBα protein, we treated these cells with IL-1β for 6 hr and analyzed whole-cell extracts by immunoblotting. IκBα protein levels were reduced in IκBα-siRNA-transfected cells irrespectively of IL-1 treatment (Figure 3B, right panel). Importantly, IκBβ protein levels were not affected by IκBα-siRNA or IL-1 treatment. Upon activation with anti-CD3, we detected the expected enhancement of IL-2 (Figure 3C) or IFN-γ (Figure 3D) gene induction by IL-1β costimulation in cells transfected with control siRNA (labeled "c"). However, IL-1-dependent enhancement was not seen in IκBα-siRNA transfected cells (labeled "α"). We conclude that IκBα is essential for effective enhancement of IL-2 and IFN-γ transcription in these cells. Taken together with the phenotype of c-Rel$^{-/-}$ primary CD4+ T cells, these observations support the proposition that naive T cells may be primed for TCR-induced cytokine gene expression by increased levels of c-Rel/IκBα complexes generated in response to proinflammatory cytokines.

### In Vivo Priming Requires c-Rel

Cells of the innate immune system participate actively in adaptive responses by enhancing antigen presentation, by providing costimulatory "second" signals via upregulation of coreceptor ligands, and by producing inflammatory cytokines (Allison, 1994; Banchereau and Steinman, 1998; Hoebe et al., 2004; Sharpe and Freeman, 2002). Our in vitro results described above suggested that inflammatory cytokines might affect T lymphocytes

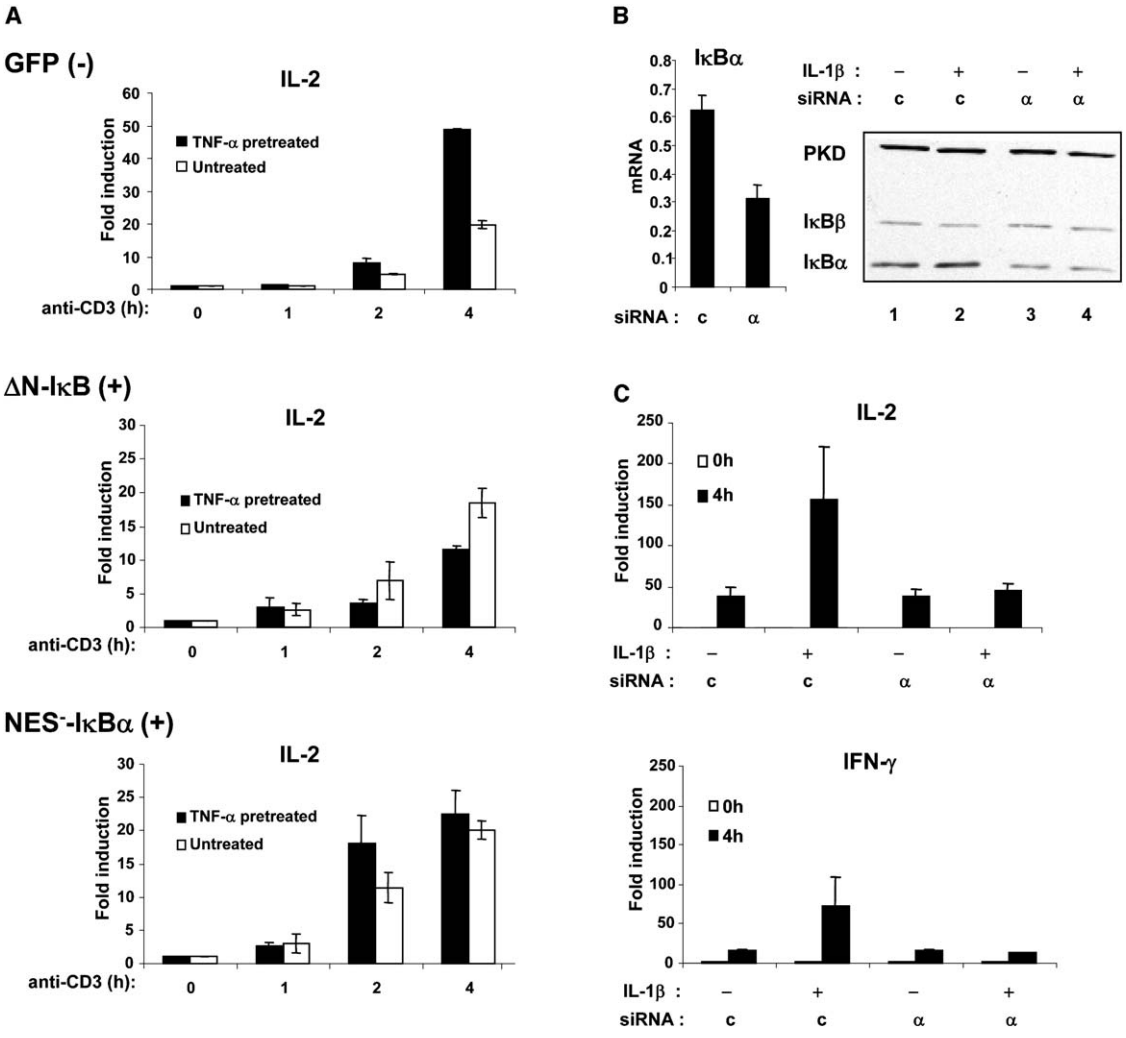


Figure 3. Enhanced Cytokine Gene Expression in Primed Cells Requires IkBα

(A) D5h3 hybridoma cells were infected with a retroviral vector that contained either a dominant-negative IκB (ΔN-IκB) or a mutated N-terminal nuclear export sequence (NES) containing IκBα (NES$^-$-IκB). Infected cells were sorted by flow cytometry to 99% purity into GFP-negative (top) and GFP-positive (middle and bottom) populations. The purified cells were left untreated or were treated with TNF-α before being activated with plate bound anti-CD3 (0.5 μg/ml) for the indicated times (*x* axis). Total RNA prepared from the cells was examined for IL-2 expression via quantitative RT-PCR as described in the Experimental Procedures section. The signals were normalized to TCRβ mRNA and represented as "Fold Induction" (*y* axis) over the basal level in cells not activated with anti-CD3. Anti-CD3-mediated activations were carried out in duplicate, and the data are an average of two independent experiments. The error bars shown represent the standard error within the duplicates.

(B) SiRNA-mediated knockdown of IκBα in primary CD4$^+$ T cells reduces priming by IL-1β. CD4$^+$ splenic T cells were electroporated with control (c) or IκBα-specific (α) siRNA followed by analysis of IκBα mRNA levels via quantitative RT-PCR (left panel). The effect on IκBα protein was examined in whole-cell extracts prepared from cells cultured in the presence or absence of IL-1β (5 ng/ml) for 6 hr. (right panel).

(C) IL-1β-treated or control cells were activated with plate-bound anti-CD3 (0.5 μg/ml) for 4 hr and IL-2 or IFN-γ mRNA expression was quantitated by RT-PCR as described. Expression levels between samples were normalized to TCRβ mRNA and expressed as "Fold Induction" (*y* axis) over the basal levels in cells not activated with anti-CD3. Results shown were obtained from two independent transfection experiments analyzed for RNA in triplicates, and the error bars represent the standard error between the two sets.

by increasing the proportion of c-Rel/IκBα complexes. To extend these observations, we determined whether in vivo activation of innate cells enhanced T cell responses by a c-Rel-dependent pathway.

We isolated spleen and lymph-node T cells from mice that had been injected with complete Freund's adjuvant (CFA) or saline (PBS) for two days, stimulated them with plate bound anti-CD3 antibodies, and assayed cytokine gene expression by quantitative RT-PCR as described above. We observed faster kinetics and higher levels of IL-2 mRNA production in T cells from normal mice injected with CFA than we did in PBS-injected controls (Figure 4A) or incomplete-Freund's-adjuvant-injected controls (Figure S2). The effect of CFA injection was substantially more pronounced on IFN-γ gene activation (Figure 4A; also Figure S3A). In contrast, there was virtually no difference in the level of cytokine gene transcription in c-Rel$^{-/-}$ T cells from either CFA-injected or

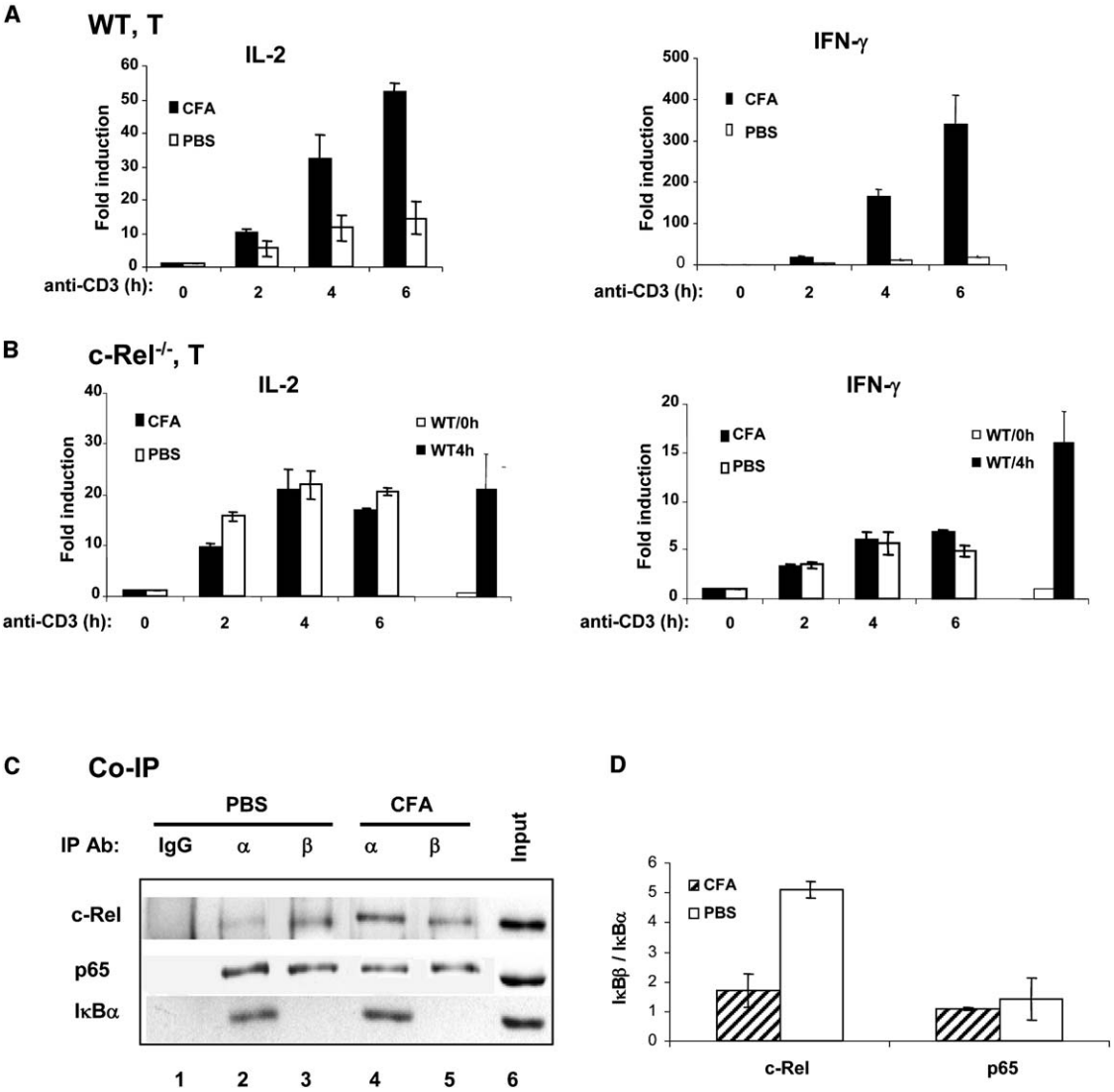


Figure 4. Adjuvant-Mediated Priming of T Cells Requires c-Rel

WT/B6 (A) or c-Rel$^{-/-}$ (B) mice were injected intraperitoneally with complete Freund's adjuvant (CFA) or saline (PBS) followed by a second injection of incomplete Freund's (IFA) and PBS, respectively, on the next day. Three days later, spleen and lymph-node (LN) T cells were activated with plate bound anti-CD3 (0.5 μg/ml), and the levels of IL-2 (left) and IFN-γ (right) mRNA were determined as described in the legend to Figure 2. RNA assays were carried out in duplicate, and error bars shown represent the standard error within the duplicate. For ease of comparison, additional bars marked WT/untreated in (B) show the level of cytokine mRNA in normal cells treated with PBS; this level is an average of 5–6 independent experiments. "Fold Induction" (*y* axis) represents the level of cytokine gene expression over the basal level in cells prior to anti-CD3 treatment. A representation of absolute mRNA levels is shown in Figures S3A and S3B. Data shown are representative of three independent experiments involving normal and c-Rel-deficient mice.

(C) WT/B6 mice were intraperitoneally injected with CFA or PBS and CD4$^+$ T cells were rapidly purified from spleen and lymph nodes in the cold. Whole-cell extracts prepared from these cells were used for coimmunoprecipiation studies (Co-IP) (left panel). The origin of extracts is noted on the top line above the figure, and the immunoprecipitating antibody is noted on the second line; IgG represents non-specific rabbit IgG, and α and β represent anti-IκBα and anti-IκBβ antibodies, respectively. Specificity and efficiency of immunoprecipitations were confirmed by immunoblotting analysis of immunoprecipitation supernatants (Figure S3C). The immunoprecipitates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes, which were probed with anti-p65/RelA, anti-c-Rel, and anti-IκBα antibodies, as indicated. The data are representative of at least three independent experiments.

(D) The proportion of p65/RelA or c-Rel bound to IκBβ compared to IκBα was quantified as described in the Experimental Procedures, and the average of three independent experiments is represented graphically. Error bars represent the standard error between experiments.

PBS-injected mice (Figure 4B). The easily detectable induction of IL-2 and IFN-γ expression in c-Rel$^{-/-}$ T cells from PBS-injected mice reconfirmed that c-Rel was not essential for inducible gene transcription. However, the priming effect of CFA was completely absent in the c-Rel-deficient T cells. In all cases we verified that CFA injection did not significantly elevate basal IL-2 or IFN-γ gene expression in the absence of anti-CD3 treat-

ment in vitro (Figures S3A and S3B show quantitation of absolute mRNA levels under these conditions). We also stained cells intracellularly for IL-2 and IFN-γ to ascertain whether increased mRNA reflected a greater proportion, or a higher level, of expression in the primed cells. For both CD4+ and CD8+ T cells, we found that cytokine levels were higher in primed cells (data not shown). These observations indicate that c-Rel is important for priming naive T cells for increased IL-2 and IFN-γ production.

To determine whether CFA injection altered the proportion of c-Rel/IκBα complexes, we isolated CD4+ T cells from the spleens of injected mice and assayed whole-cell extracts for c-Rel and p65/RelA association with IκBα and IκBβ. We found comparable levels of p65/RelA associated with IκBα or IκBβ in T cells from control (PBS-injected) or CFA-injected mice (Figure 4C). However, more c-Rel was associated with IκBα in CFA-injected mice (Figure 4C; quantitated in Figure 4D). In each case we verified that the IκB antibody quantitatively depleted the appropriate protein by analyzing supernatants from the coimmune precipitation experiments (Figure S3C). These results are consistent with the hypothesis that CFA priming in vivo resulted in a shift of c-Rel from IκBβ- to IκBα-associated complexes.

c-Rel-deficient innate immune cells produce lower levels of certain cytokines (Boffa et al., 2003; Grigoriadis et al., 1996), such as interleukin 12 (Mason et al., 2002; Ouaaz et al., 2002; Sanjabi et al., 2000). Although a requirement for c-Rel for TNF-α or IL-1β production has not been directly demonstrated, the absence of T cell priming by CFA in c-Rel-deficient mice may be due to ineffective activation of innate immune cells in these mice. To address this, as well as determine whether TNF-α and IL-1β could prime T cells in vivo, we assayed IL-2 and IFN-γ mRNA expression in T cells isolated from mice treated with TNF-α plus IL-1β.

Total T cells were isolated from lymph nodes and spleens of mice injected with these cytokines, or with PBS as a control, and activated in vitro with anti-CD3 antibodies. We found significantly higher levels of IL-2, as well as IFN-γ, mRNA in T cells from TNF-α-, IL-1β-treated mice (Figure 5A). The difference in IFN-γ mRNA between cytokine-injected and control mice was not as large as that seen with CFA treatment. It is possible that innate cells from CFA-injected animals produced additional cytokines, such as IL-12, which enhanced IFN-γ production in T cells (Curtsinger et al., 1999; Schmidt and Mescher, 2002). Unlike the effect in normal mice, T cells from c-Rel$^{-/-}$ mice injected with TNF-α and IL-1β did not show elevated IL-2 or IFN-γ gene transcription in comparison to control (PBS-injected) c-Rel$^{-/-}$ mice (Figure 5B). We confirmed that the increases seen in TNFα+IL-1β-primed mice were also reflected in the absolute levels of IL-2 and IFN-γ mRNA (Figures S4A and S4B).

To directly examine the priming effect on CD4+ T cells, we assayed IL-2 and IFN-γ expression after CD3 activation of purified CD4+ cells fom normal mice, or DO11 TCR transgenic mice on a RAG2-deficient background, that had previously been primed with CFA or with TNFα plus IL-1β. In both cases, we observed increased IL-2 and IFN-γ mRNA in cells from treated mice (Figures S5 and S6).

CD4+ T cells from TNF-α-, IL-1β injected mice also showed a shift of c-Rel to IκBα-associated complexes (Figure 5C), although the magnitude of the effect was smaller than that seen with CFA. Immunoprecipation efficiency and specificity was confirmed by assaying the immunoprecipitation supernatants (Figure S4C). We conclude that pro-inflammatory cytokines prime T cells in vivo by altering the proportion of c-Rel/IκBα complexes. The altered proportion of c-Rel associated with either IκBβ or IκBα probably indicates a change in the amount of c-Rel/IκBα complexes because total c-Rel, or p65, levels did not vary significantly in various cell preparations.

### Cytokine Priming Is a Feature of Naive T Cells

In published reports, the difference in IL-2 or IFN-γ production by normal or c-Rel$^{-/-}$ T cells in response to high levels of anti-CD3 or anti-CD3 plus anti-CD28 (CD3+CD28) varies considerably. With one exception, however, c-Rel$^{-/-}$ T cells always produce less cytokine than normal cells (Hilliard et al., 2002; Kontgen et al., 1995; Liou et al., 1999; Rao et al., 2003). In contrast, pharmacologically activated c-Rel$^{-/-}$ and normal T cells make comparable levels of IL-2 or IFN-γ (Grigoriadis et al., 1996; Kontgen et al., 1995). In our study, low-level CD3 stimulation of splenic T cells resulted in no more than 2- to 2.5-fold-reduced cytokine mRNA in c-Rel$^{-/-}$ splenic T cells compared to normal cells. These observations suggest that IL-2, or IFN-γ, gene transcription can be activated by c-Rel-dependent or c-Rel-independent pathways. Indeed, differential activation of the two pathways may be part of the explanation for the wide variation that earlier studies noted between normal T cells and c-Rel$^{-/-}$ T cells.

A hallmark of effector T cells, compared to naive T cells, is their rapid and elevated expression of cytokine genes in response to TCR signals. It was therefore of interest to determine whether c-Rel was required for elevated IL-2 and IFN-γ transcription in effector cells, as well as to determine whether these cells could be "primed" by TNF-α and IL-1β. To do this, we expanded splenic CD4+ T cells from normal, or c-Rel$^{-/-}$, mice by treatment with immobilized anti-CD3 plus soluble anti-CD28 in the presence of rIL-2 and assayed cytokine gene expression in response to TCR signals.

As expected, IL-2 expression in CD4+ splenic T cells was significantly enhanced by CD28 costimulation at two concentrations of activating antibodies (Figure 6A). Interestingly, the level of IL-2 mRNA expression in response to anti-CD3 and anti-CD28 (0.5 μg/ml) was similar to that produced in response to 0.5 μg/ml CD3 plus TNF-α and IL-1β priming (Figure 2). Thus, cytokine costimulation compared favorably with CD28 costimulation, at least under conditions of limiting TCR stimulation. CD28-mediated enhancement was entirely c-Rel dependent (Figure 6A, left panel), consistent with earlier observations that the CD28 response element is a target of c-Rel (Himes et al., 2000; Rao et al., 2003). In contrast, although IL-2 mRNA was expressed at much higher levels in CD3 blasts and remained CD28 costimulation dependent, we found that c-Rel$^{-/-}$ T cells responded no differently than normal CD4+ T cells (Figure 6B). These observations indicate that the c-Rel require-

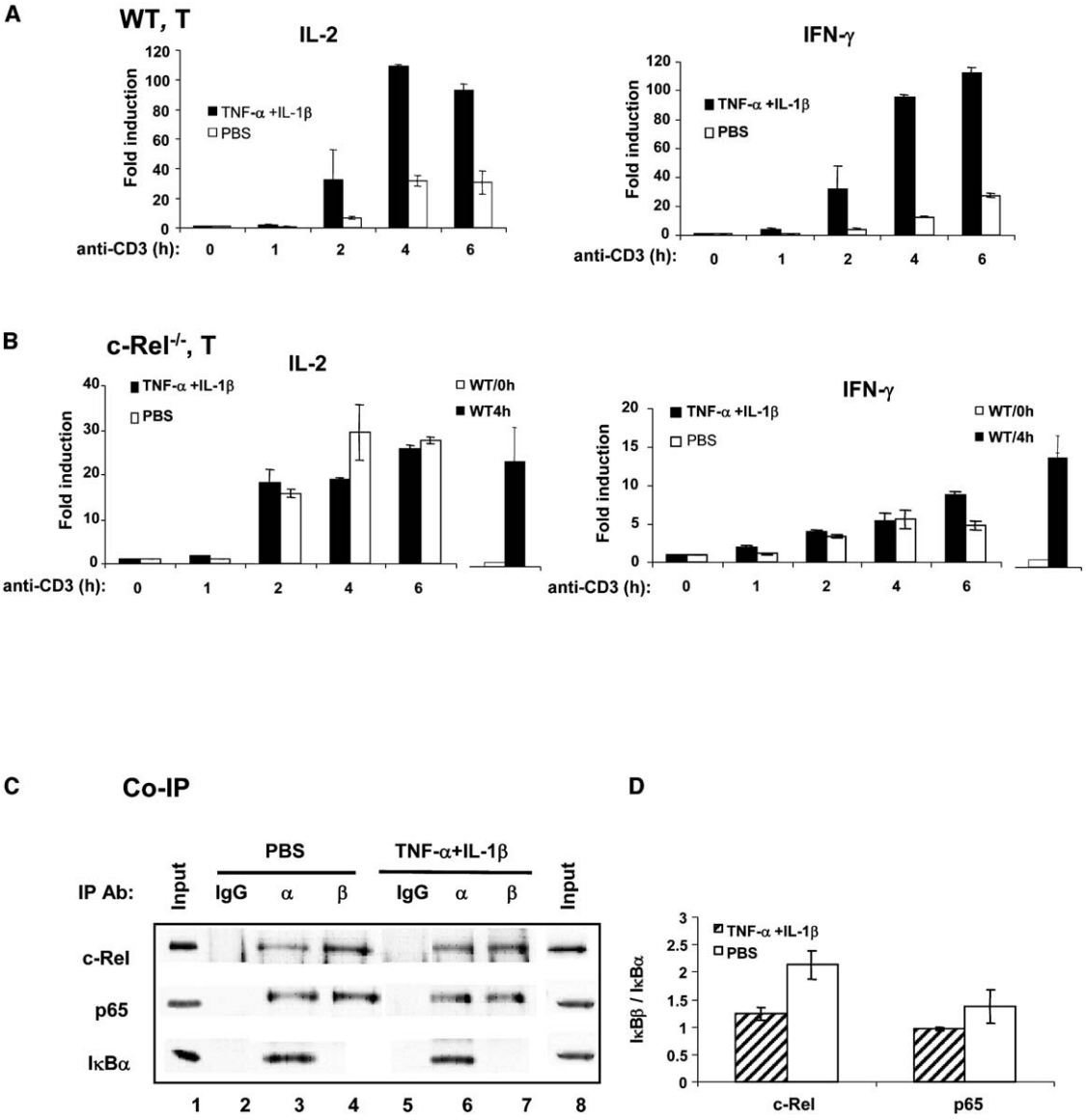


Figure 5. TNF-α- and IL-1β-Mediated Priming of T Cells is c-Rel Dependent

WT/B6 (A) or c-Rel$^{-/-}$ (B) mice were injected intraperitoneally with adjuvant cytokines, TNF-α and IL-1β, or PBS, as described in the Experimental Procedures section. IL-2 and IFN-γ production was determined by quantitative real-time RT-PCR as described previously. RNA assays were carried out in duplicate, and error bars shown represent the standard error within the duplicate. For ease of comparison, additional bars marked WT/untreated in (B) show the level of cytokine mRNA in normal cells treated with PBS, and this level is an average of 5–6 independent experiments. "Fold Induction" (*y* axis) represents the level of cytokine gene expression over basal levels in cells prior to anti-CD3 treatment. A representation of absolute mRNA levels is shown in Figures S4A and S4B. The data are representative of at least three independent experiments with normal and c-Rel-deficient mice. (C) CD4$^+$ T cells were rapidly purified from spleen and lymph nodes of cytokine-primed or control mice in the cold and immunoprecipitated as described in (C) of Figure 4. Immunoprecipitation, efficiency, and specificity were confirmed by analysis of supernatants (Figure S4C). Co-immunoprecipitated proteins were visualized by immunoblotting after separation by SDS-PAGE. Data shown are representative of three independent experiments. (D) The proportion of p65/RelA or c-Rel bound to IκBβ compared to IκBα was quantified as described in the Experimental Procedures, and the average of three independent experiments is represented graphically. As discussed in the text, because the levels of c-Rel and p65 do not change with TNFα or IL-1β treatment, the altered proportions reflect a change in the amount of c-Rel/IκBα complexes. Error bars represent the standard error between experiments.

ment for CD28 costimulation of IL-2 gene expression is a property of naive cells only.

Although IFN-γ induction was much less dependent on CD28 costimulation, we again noted differences between normal and c-Rel-deficient cells only in freshly isolated splenic preparations (Figure 6A, right panel). As observed for IL-2, CD3 blasts from normal or c-Rel$^{-/-}$ T cells responded robustly to even very low concentrations of anti-CD3 alone or various combinations of anti-CD3 and anti-CD28 (Figure 6B). These data are represented in terms of absolute mRNA levels in Figure S7. Finally, we treated blast cells with TNF-α and IL-1β prior

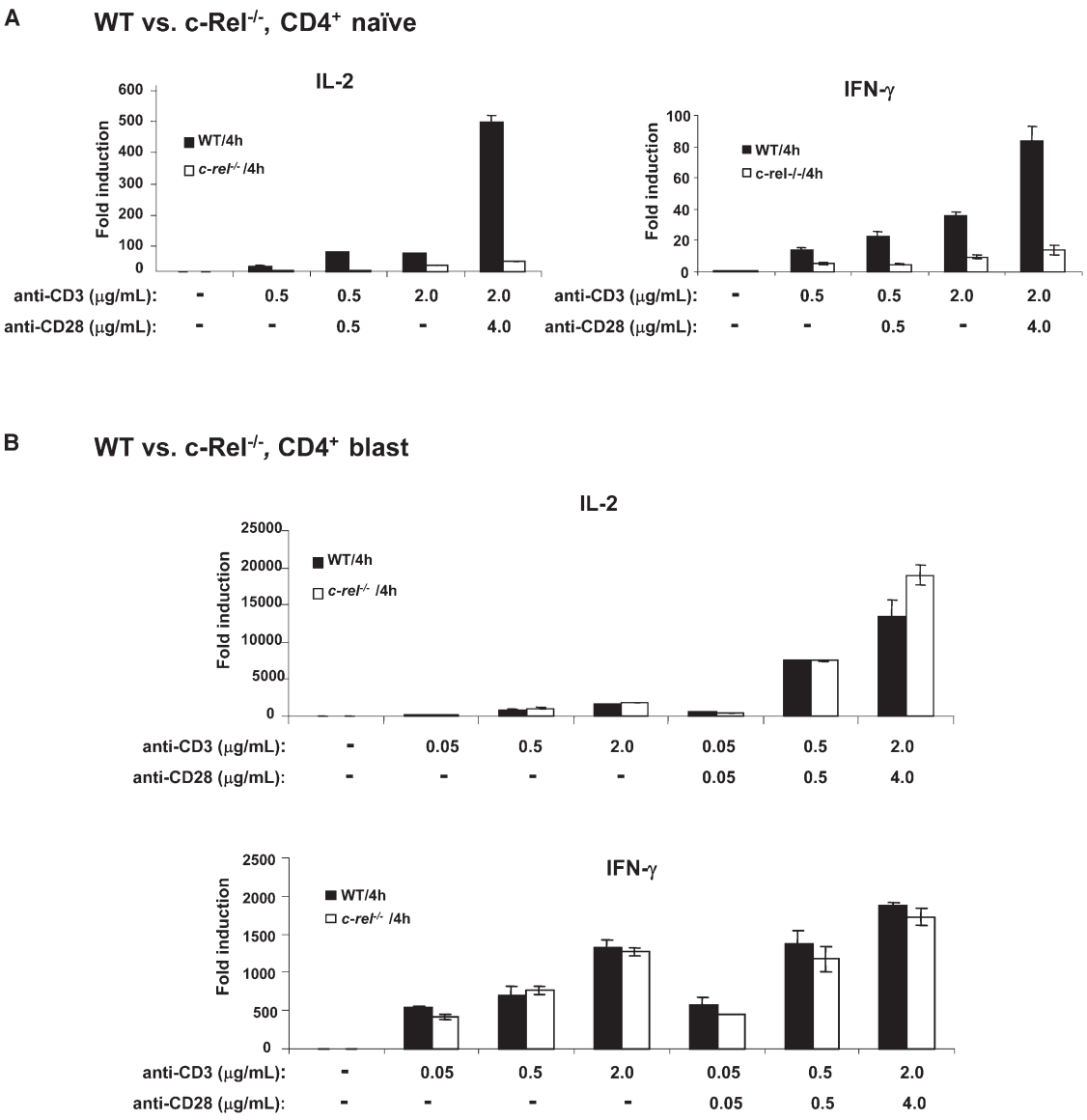


Figure 6. c-Rel-Dependent Priming Is a Feature of Naive CD4+ T Cells

(A) CD4+ T cells ere purified from the spleen of WT/B6 or c-Rel$^{-/-}$ mice and activated with various levels of immobilized anti-CD3 or with anti-CD3 plus anti-CD28 antibodies for 4 hr. RNA prepared from these cells was assayed for IL-2 and IFN-γ gene expression by quantitative real-time RT-PCR. "Fold Induction" (*y* axis) represents the level of cytokine gene expression over basal levels in cells prior to anti-CD3 treatment. A representation of absolute mRNA levels is shown in Figure S7. The data shown are representative of three independent experiments done in triplicate.

(B) CD4+ T cell blasts were prepared as described in the Experimental Procedures section from normal and c-Rel$^{-/-}$ mice. After overnight rest in fresh medium without IL-2, the cells were activated with immobilized anti-CD3 or with anti-CD3 plus anti-CD28, as indicated, for 4 hr. RNA prepared from these cells was assayed for IL-2 and IFN-γ expression by quantitative real-time RT-PCR. The data are representative of three independent experiments done in duplicate, with error bars representing the standard error within duplicates.

to TCR activation; however, this had no effect on IL-2 or IFN-γ expression (data not shown). This was not due to faulty TNF-α (or IL-1) signaling because IκBα degradation in response to these cytokines occurred normally in wild-type and c-Rel-deficient cells. We conclude that c-Rel-dependent priming of IL-2 and IFN-γ gene expression is a unique feature of naive T cells. Not only are effector T cells no longer sensitive to this pathway, but high-level cytokine gene expression in these cells is c-Rel independent.

## Discussion

Considerable experimental evidence supports the model in which three signals are required to optimally activate naive T cells to proliferate. These are (1) signals initiated at the T cell receptor, (2) the CD28 coreceptor, and (3) the presence of inflammatory cytokines. Although MHC molecules that serve as ligands for the TCR are constitutively expressed on APC (but subject to further modulation), upregulation of c-stimulatory molecules

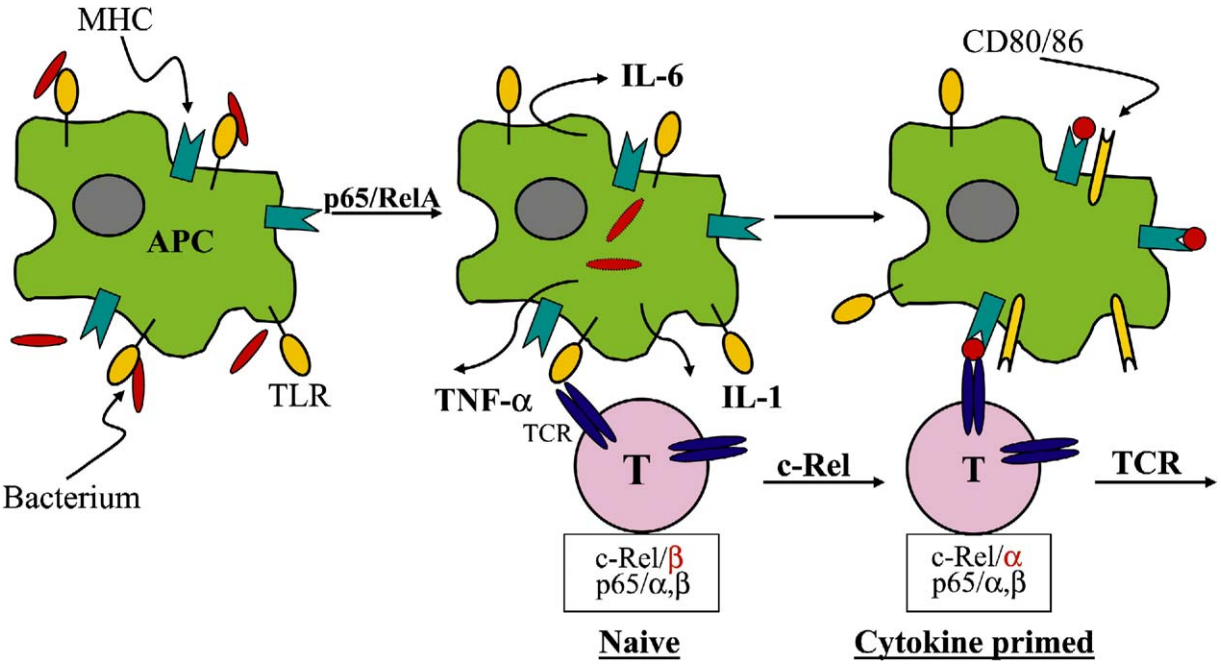


Figure 7. A Model for c-Rel-Dependent Priming of Naive T Cells by Inflammatory Cytokines

In the cytosol of naive T cells, the NF-κB family member c-Rel is complexed primarily to the inhibitor IκBβ, whereas p65/RelA is complexed to IκBα and IκBβ (lower line, labeled Naive). Pathogen recognition by antigen-presenting cells leads, via NF-κB activation, to secretion of pro-inflammatory cytokines such TNF-α, IL-1, and IL-6, as well as upregulation of costimulatory molecules CD80 and CD86. These cytokines induce IκBα turnover with a consequent shift of c-Rel to IκBα-associated complexes in primed T cells (lower line, labeled "Cytokine Primed"); c-Rel/IκBα complexes are readily targeted by TCR-initiated signals, leading to faster and higher levels of IL-2 and IFN-γ gene expression. Costimulatory signals transmitted by CD28/CD80, 86 interactions also target c-Rel by increasing IκBβ degradation. Both mechanisms contribute to efficient c-Rel induction and, therefore, increased efficiency of IL-2 and IFN-γ gene transcription. We propose that c-Rel serves as a danger sensor in T cells.

CD80 and CD86, which are CD28 coreceptor ligands, and pro-inflammatory cytokine gene expression require APC activation by Toll-like receptors (Akira and Takeda, 2004; Beutler, 2004). The latter signals ensure that T cell activation in vivo is largely restricted to situations where innate immunity has already been triggered by the incoming antigen. Our observations implicate c-Rel, an NF-κB family member, as an essential component of the communication between innate immune cells and primary T cell responses.

The mechanism we propose is based on our earlier observation that resting T cells contain predominantly c-Rel/IκBβ complexes. This c-Rel is refractory to release by TCR-initiated signals, which do not target IκBβ efficiently. Thus, the only way to induce nuclear c-Rel protein for cytokine (IL-2 and IFN-γ) gene expression is via new c-Rel gene transcription and translation. We now show that pro-inflammatory cytokines generate c-Rel/IκBα complexes by increasing IκB turnover and export of nuclear c-Rel by newly synthesized IκBα (Figure 7). These primed T cells are much more responsive to TCR signals, which readily target IκBα, resulting in rapid mobilization of c-Rel to the nucleus and c-Rel-dependent gene expression. Lack of inflammatory cytokine-mediated priming in c-Rel-deficient T cells in vitro, and in vivo, underscores the importance of c-Rel in this process.

It is interesting to note that the other form of danger sensing, via upregulation of costimulatory molecules, also targets rapid c-Rel nuclear translocation. CD28-initiated costimulatory signals have been extensively studied, but a qualitative difference between one (TCR alone) and two signals that accounts for increased IL-2 production has been difficult to discern (Kane et al., 2002). Indeed, recent studies conclude that the difference between CD3 alone versus CD3 plus CD28 signals may be a quantitative one (Diehn et al., 2002; Feske et al., 2001). One aspect of NF-κB biology, however, appears to be qualitatively different. Specifically, CD3 plus CD28 signals target both IκBα and IκBβ in cell lines as well as primary cells (Harhaj et al., 1996; Zhou et al., 2002). In light of our observation that most of the c-Rel in unactivated T cells is IκBβ bound, the CD28 costimulus results in c-Rel activation by the classical, post-translational mechanism of NF-κB activation. Thus, both CD28 costimulation and proinflammatory cytokines have the same effect on c-Rel in naive T cells; they help activate c-Rel rapidly in response to antigen and thus enable faster and higher levels of IL-2 and IFN-γ gene expression.

Recent studies underscore the fact that upregulation of costimulatory molecules (UCM) is not sufficient to provide effective costimulation (Hoebe et al., 2003; Kaisho et al., 2001; Pasare and Medzhitov, 2004). Specifically, UCM and TNF-α production are both impaired in Trif-deficient macrophages in response to

LPS, whereas only TNF-α production is diminished in Myd88-deficient macrophages. However, compared to normal mice, both mutants have reduced T cell responses. Because UCM was unaltered in Myd88$^{-/-}$ cells, the authors concluded that inflammatory cytokine-induced T cell priming is essential for productive T cell activation, regardless of the status of costimulatory molecules. In the framework of the model proposed in this paper, we suggest that the lack of responsiveness in Myd88-deficient mice may be due to inefficient c-Rel/IκBα complex formation in T cells in the absence of inflammatory cytokines.

Most of our analyses were carried out under conditions of low but continuous inflammatory-cytokine treatment. However, initial experiments showed that the shift of c-Rel to IκBα also occurred with a short, acute pulse of TNF-α (Figure 1). Moreover, c-Rel/IκBα complexes were present as long as 6 hr after removal of TNF-α, at a time when IκBα and IκBβ levels had been restored to starting levels. These observations indicate that the "memory" of prior inflammatory cytokine activation, in the form of c-Rel/IκBα complexes, lasts quite a long time. During this period the cells remain hyperresponsive to TCR signals, and it is tempting to speculate that such primed cells may be targets for stimulation by autoantigens. Such aberrant, CD28-independent, T cell activation could result in a breakdown of self-tolerance. Although we have only explored inflammatory cytokines in this study, other IκB-degrading signals are likely to have the same effect. To this end, we found that low-level CD3 activation, which had no measurable effect on cytokine gene transcription or NF-κB activation, also generated c-Rel/IκBα complexes and primed cells for subsequent TCR-mediated activation (D.B., unpublished observation).

Lastly, we found that IL-2 and IFN-γ gene induction in primary CD4$^+$ T cell blasts was c-Rel independent. Moreover, these cells could no longer be primed with TNF-α plus IL-1β to increase cytokine gene transcription. Thus, c-Rel-dependent cytokine gene expression and the ability to be primed are features of naive T cells only. These observations are consistent with the view that c-Rel plays a crucial role as a danger sensor in T cells. It is well established that naive cells are particularly refractory for IL-2 or IFN-γ gene transcription (Grogan et al., 2001; London et al., 2000; London et al., 1999), presumably to minimize full-fledged T cell activation unless there is a real need. We suggest that the dampened response is, in part, due to the requirement for c-Rel for robust transcription of these genes, although other NF-κB family members can partially compensate for the absence of c-Rel. However, because c-Rel is associated mainly with IκBβ in naive T cells, it is not easily inducible. The difficulty of c-Rel induction can be circumvented in (at least) two ways: by provision of a costimulatory (CD28) signal that increases IκBβ degradation and by pro-inflammatory cytokine signals that shift c-Rel to IκBα-associated complexes. Both means of increasing c-Rel responsiveness in T cells are the result of innate immune cells' "danger-sensing" mechanisms that are mediated by Toll-like receptors. Effector cells that arise as a result of the primary response have already been tested for the "need" to respond and therefore may not require additional signals for cytokine gene induction. This is reflected in a reduced requirement for c-Rel.

## Experimental Procedures

### Mice

All mice used for the experiment were 6–8 weeks old. C57BL6 (B6) and DO11.TCR Tg/Rag$^{-/-}$ mice were purchased from Taconic Farms (Germantown, NY), and c-Rel$^{-/-}$ mice were generously provided by the lab of Dr. Hsiou-Chi Liou (Cornell University Medical College, NY). These mice were bred in the Brandeis University Animal Care Facility, and all in vivo studies were done in compliance with the guidelines of our facility and the ACUC.

### Cell Cultures and Reagents

D5h3 CD4$^+$ T cell hybridoma cells were maintained in DMEM (Gibco Invitrogen, Carlsbad, CA) medium supplemented with 10% FBS, 1% penicillin and streptomycin, 1 mM L-glutamine, and 50 μM β-mercaptoethanol. All antibodies were purchased from Santa Cruz Biotechnology, CA, except anti-CD3 mAb (BD Pharmingen, San Diego, CA). Murine TNF-α and IL-1β cytokines for in vitro treatment were obtained from R and D Systems (Mineapolis, MN) and Roche Biochemical (Indianapolis, IN), respectively, and cytokines for intraperitoneal injections were from Peprotech (Rocky Hill, NJ). Complete and incomplete Freund's (CFA/IFA) were purchased from Difco Laboratories (Detroit, MI).

### Intraperitoneal Injections and Cell Isolation

200 μl of an emulsion of CFA/IFA in PBS was injected into each of 5–6 age-matched littermates per experiment, and this was followed by another dose of IFA on the next day. For adjuvant cytokines, 250 ng of TNF-α and 500 ng of IL-1β was injected on three consecutive days. These cytokines were reconstituted in PBS without carrier bovine serum albumin. PBS alone was used as a negative control for all experiments. The animals were sacrificed on day 5, and single-cell suspensions from spleen and lymph nodes (LNs) were treated with CD4$^+$ L3T4 microbeads and purified with MACS magnetic columns (Miltenyi Biotech, Auburn, CA.) as specified by the manufacturer. This protocol yielded cells with >95% purity as assessed by flow cytometry. Negative selection of total T cells was carried out with the Pan-T isolation kit from Miltenyi Biotech.

### Primary T Cell Activation

Freshly isolated CD4$^+$ T cells were treated continuously with TNF-α (5 ng/ml), IL-1β (5 ng/ml), or TNF-α and IL-1β for 6 hr or as indicated. These cells were collected by centrifugation and plated at a density of 1 × 10$^6$/ml in six-well plates precoated with 0.5 μg/ml of anti-CD3 mAb (BD Pharmingen). Cells were harvested at various times for RNA and protein analyses. All activation assays were carried out in duplicate for each time point.

### Preparation of CD3 Blast Cells

Purified CD4$^+$ T cells were activated with anti-CD3 (1 μg/ml), anti-CD28 (2 μg/ml, soluble), and recombinant human (rh) IL-2 (10 ng/ml, BD Pharmingen) for 2 days followed by a further 2 days of growth rhIL-2 to generate blasts. These cells were washed, rested overnight in media lacking rhIL-2, and activated with plate bound anti-CD3 and soluble anti-CD28 at the concentrations as indicated.

### RNA Isolation and Real-Time Reverse-Transcription PCR

RNA was isolated by with the RNeasy Mini kit (Qiagen, Valencia, CA.) and 200–500 ng was used for reverse transcription (RT) with Superscript III reverse transcriptase (Invitrogen). The RT reaction was diluted 1:10 and 1–2 μl was used for real-time RT-PCR with SYBR-Green I detection via a Rotor-Gene 2000 (Corbett Research, USA). Appropriate standards were generated for the IL-2 and IFN-γ genes with RNA prepared from DC27.10 CD4$^+$ T hyridoma cells and primary CD8$^+$ T cells, respectively, that were activated with immobilized anti-CD3 (2 μg/ml) and soluble anti-CD28 (2 μg/ml) for 4 hr. The Ct values of the samples were determined from the standard curve by a method similar to that used by Ramos-Payan et al. (Ramos-Payan, 2003). IL-2 and IFN-γ mRNA levels were normalized to that of TCR Cβ2 to get the "absolute" mRNA. For "Fold Induction,"

the values of absolute mRNA were normalized to the value in CD3-treated controls.

Primer sequences:
IL-2
FP: 5′-CCTGAGCAGGATGGAGAATTACA-3′
RP: 5′-TCCAGAACATGCCGCAGAG-3′
IFN-γ
FP: 5′-GCGTCATTGAATCACACCTG -3′
RP: 5′-GACCTCAAACTTGGCAATACTCATG-3′
C-beta
FP: 5′-GCCGAGCAGACTGTGGAATC-3′
RP: 5′-GGAATTTTTTTTCTTGACCATGGCC-3′
IκBα
FP: 5′-GACGGAGACTCGGTTCCTGC-3′
RP: 5′-GTGGAGTGGAGTCTGCTGC-3′

### Extracts and Immunoprecipitations

Nuclear and whole-cell extracts (WCE) were prepared as described (Tam et al., 2001, Xu et al., 2003). WCE (50–100 μg) was treated with anti-IκBα (sc-371, Santa Cruz) or anti-IκBβ (sc-945, Santa Cruz) antibodies, and the immunoprecipitates were collected on protein A agarose beads (Roche Pharmaceuticals). After fractionation through 8% SDS-PAGE, proteins were transfered to nitrocellulose membranes, which were probed with anti-c-Rel or anti-p65 antibodies. Detection was carried out with the West pico chemiluminescent kit (Pierce/Fisher). The supernatants were precipitated with acetone and checked via immunoblotting to ensure complete depletion of the respective proteins.

### Electrophoretic Mobility-Shift Assay

Binding assays were performed with T cell nuclear extracts prepared as above. Probes used for an electrophoretic mobility-shift assay (EMSA) were the H2KκB element and the μE3 binding site from the immunoglobulin heavy-chain gene enhancer. All probes and conditions for the EMSA have been previously described (Venkataraman et al., 1995).

### Gel Quantitation

The Quantity One Software program (Biorad) was used to quantitate band intensities in Western blots. As per the software usage protocol, tiny boxes were made around each band of interest, and a same-sized box in close proximity determined the background. This background number was automatically subtracted by the program from the raw numbers assigned for each band to give the net band intensities. The net band intensity of c-Rel associated with IkBβ was then divided by that of c-Rel associated with IκBα. IκB-associated p65 was treated similarily. The ratios from three independent experiments were averaged to generate Figures 4D and 5D; the error bars represent the standard error between experiments.

### MSCV Retroviral Infection

The construct, dominant-negative IκB (DN-IκB) expressing GFP-MSCV retroviral vector, was a generous gift from Dr. B. Horwitz (Department of Pathology, Brigham and Women's Hospital, MA). The N terminal NES-mutated IκB-α (NES-IkB-α) fragment (Tam et al., 2001) from a pc-DNA3 vector was cloned into MSCV-GFP vector into the ECoR1/BamH1 sites. These constructs were transfected into packaging cell lines, BOSC 23 by the $CaCl_2$/HBS method of Van Parjis et al. (Van Parijs et al., 1999), to generate the virus. Viral supernatants collected 48 hr and 72 hr after transfection were used to spin-infect D5h3 hybridoma cells. After 48 hr, the $GFP^+$ and $GFP^-$ cells were sorted to 99% purity by FACS (Becton Dickinson) for activation and RNA analyses.

### SiRNA Nucleofection

Splenic $CD4^+$ T cells were isolated by positive selection and electroporated with an AMAXA nucleofector device according to the manufacturer's protocol with the following modifications. After purification, cells were rested for approximately 1 hr at 37°C and then transfected with 3.5 μg of control or IκBα siRNA (Dharmacon, Chicago, IL). Approximately $2 \times 10^6$ cells were used per electroporation. Overall cell viability after electroporation was approximately 75%. The cells were allowed to recover for 3 hr before being treated with IL-1β (ng/ml) for 6 hr, followed by anti-CD3 treatment (0.5 μg/ml) for an additional 4 hr. Total RNA was used to quantitate IL-2 and IFN-γ gene expression by RT-PCR as described above.

### Supplemental Data

Eight supplemental figures are available with this article online at http://immunity.com/cgi/content/full/23/4/445/DC1/.

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